

Fluorescein isothiocyanate labeled, magnetic nanoparticles conjugated D-penicillamine-anti-metadherin and in vitro evaluation on breast cancer cells

Avaliação do isotiocianato de fluoresceína marcado, das nanopartículas magnéticas conjugadas da D-penicilamina antimetaderina e in vitro nas células do câncer de mama

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Abstract

Silane modified magnetic nanoparticles were prepared after capped with silica generated from the hydrolyzation of tetraethyl orthosilicate (TEOS). Amino silane (SG-Si900) was added to this solution for surface modification of silica coated magnetic particles. Finally, D-penicillamine (D-PA)-anti-metadherin (anti-MTDH) was covalently linked to the amine group using glutaraldehyde as cross-linker. Magnetic nanoparticles were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), vibrating sample magnetometer (VSM), and atomic force microscopy (AFM). AFM results showed that particles are nearly monodisperse, and the average size of particles was 40 to 50 nm. An amino acid derivative D-PA was conjugated anti-MTDH, which results the increase of uptaking potential of a conjugated agent, labelled fluorescein isothiocyanate (FITC) and then conjugated to the magnetic nanoparticles. *In vitro* evaluation of the conjugated D-PA-anti-MTDH-FITC to magnetic nanoparticle was studied on MCF-7 breast cancer cell lines. Fluorescence microscopy images of cells after incubation of the sample were obtained to monitor the interaction of the sample with the cancerous cells. Incorporation on cells of FITC labeled and magnetic nanoparticles conjugated D-PA-anti-MTDH was found higher than FITC labeled D-PA-anti-MTDH. The results show that magnetic properties and application of magnetic field increased incorporation rates. The obtained D-PA-anti-MTDH-magnetic nanoparticles-FITC complex has been used for *in vitro* imaging of breast cancer cells. FITC labeled and magnetic nanoparticles conjugated D-PA-anti-MTDH may be useful as a new class of scintigraphic agents. Results of this study are sufficiently encouraging to bring about further evaluation of this and related compounds for ultraviolet magnetic resonance (UV-MR) dual imaging.

Keywords: Fe₃O₄ magnetic nanoparticles, D-penicillamine, Anti-Metadherin, fluorescein isothiocyanate (FITC), MCF-7.

Resumo

Nanopartículas magnéticas modificadas de silano foram preparadas após serem tampadas com sílica criada da hidrolização do ortossilicato de tetraetil (TEOS). Aminossilano (SG-Si900) foi adicionado à solução para modificação da superfície da sílica revestida por partículas magnéticas. Por fim, a D-penicilamina (D-PA)-antimetaderina (anti-MTDH) foi covalentemente ligada ao Grupo da Amina, utilizando glutaraldeído como ligante cruzado. As nanopartículas magnéticas foram caracterizadas pela microscopia eletrônica de varredura (MEV), difração de raios X (DRX), magnetômetro de amostra vibrante (MAV) e microscopia de força atômica (MFA). Os resultados da MFA mostraram que as partículas estão quase monodispersas e o tamanho médio das partículas era de 40 a 50 nm. Um derivado aminoácido da D-PA foi conjugado como anti-MTDH, que resulta no aumento do potencial de absorção de um agente conjugado, isotiocianato de fluoresceína marcado (FITC) e depois conjugado às nanopartículas magnéticas. A avaliação *in vitro* da D-PA-anti-MTDH-FITC conjugada à nanopartícula magnética foi estudada em linhagens das células do câncer de mama MCF-7. As imagens da microscopia de fluorescência das células após a incubação da amostra foram obtidas para monitorar a interação da amostra com as células cancerígenas. A incorporação nas células do FITC marcado e das nanopartículas magnéticas conjugadas de D-PA-anti-MTDH foi encontrada superior ao FITC marcado D-PA-anti-MTDH. Os resultados mostram que as propriedades magnéticas e a aplicação do campo magnético aumentaram as taxas de incorporação. O complexo D-PA-anti-MTDH das nanopartículas magnéticas do FITC foi utilizado para a visualização *in vitro* das células de câncer de mama. O FITC marcado e as nanopartículas magnéticas conjugadas em D-PA-anti-MTDH podem ser úteis como uma nova classe de agentes cintilográficos. Os resultados deste estudo favorecem a realização de futuras avaliações para este e outros compostos relacionados para a visualização com técnicas de dupla imagem da ressonância magnética ultravioleta.

Palavras-chave: nanopartículas magnéticas Fe₃O₄, D-penicilamina, antimetaderina, isotiocianato de fluoresceína, MCF-7.

Introduction

In recent years, magnetic nanoparticles have attracted much attention due to their unique magnetic properties and widespread application in cell separation^{1,2}, drug delivery^{3,4}, magnetic resonance image (MRI) techniques⁵, cancer diagnosis and treatment⁶⁻⁹. These ferrofluids can be directed to magnetic area due to their magnetic properties¹⁰⁻¹².

Biomedical applications have increased the interest of magnetic nanoparticles into silica. The nontoxic silica is an ideal coating material because of its capability form extensive cross-linking, which leads to an inert outer shield. Silanized nanocomposites are stable in a wide range of biological environments. They are biocompatible and can also be easily activated to provide new functional group.

D-Penicillamine (D-PA) is an aminothiols and a powerful chelating agent¹³. Penicillamine is largely used in medicine in rheumatoid arthritis, Wilson's disease for the removal of copper, and in heavy metal poisoning^{16,17}. Penicillamine is a pharmaceutical of chelator class. The pharmaceutical form is D-penicillamine. Like L-penicillamine, it is highly toxic¹⁸.

Metadherin is a type 2 transmembrane protein, in which its overexpression was first described in breast cancers. It plays an important role in the metastasis of breast cancers into the lungs as a secondary site of development. Metadherin is located in a small region of human chromosome 8, and it seems to be crucial to cancer's spread or metastasis since it helps tumor cells to tightly stick to blood vessels in distant organs. The gene also makes tumors more resistant to the powerful chemotherapeutic agents normally used to wipe out the deadly cells.

Antibodies reactive to the lung-homing domain of metadherin and siRNA-mediated knockdown of metadherin expression in breast cancer cells inhibited experimental lung metastasis, indicating that tumor cell metadherin mediates localization at the metastatic site^{19,20}.

Fluorescein isothiocyanate (FITC) is the original fluorescein molecule functionalized with an isothiocyanate reactive group (-N=C=S). The isothiocyanate group reacts with amino terminal and primary amines in proteins. It has been used for labeling proteins including antibodies and lectins^{21,22}. Anti-MTDH conjugated D-penicillamine was labeled with FITC using the amine group.

A based-novel antibody and D-penicillamine, magnetic nanoparticle conjugated fluorescent complex for in vitro imaging of breast cancer cells was reported here.

Material and Methods

Materials

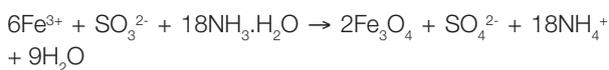
All reagents were commercially available and analytical grade. Anti-metadherin (100 mg/400mL) was purchased from Zymed. D-PA and FITC were purchased from Aldrich Chemical Co., and other chemicals were supplied from

Merck Chemical Co. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection.

Formation and Surface modification of Magnetic Nanoparticles

Synthesis of core-shell (Fe₃O₄-SiO₂) magnetic nanoparticles

Silica coated magnetic nanoparticles have been prepared by partial reduction of Fe³⁺ ion with sodium sulfide under nitrogen atmosphere. While Fe₃O₄ magnetite nanoparticles have been formed at the first step of the reaction, they were coated with silica at the second one.



Surface modification of silica coated magnetic particles with amino silane

Trialkoxysilylalkyl substitute polymethylene diamine compounds, like SG-Si900 (N-[3-(trimethoxyoxysilyl)propyl]-ethylenediamine), are used for surface coating of inorganic materials.

Glutaraldehyde Conjugation of Magnetic Nanoparticles

Determination of magnetic particles properties

The Scanning Electron Microscope (SEM) (Phillips XL-30 S FEG) was taken to determine surface morphology and size of magnetic particles. Since the samples should be dry for imaging, sample was dispersed in an evaporating solvent (methanol) after being washed with ethanol-water mixture for three times in this study. Samples were taken with micropipette and put on the steel plates for SEM measurements. They were followed five minutes after methanol treated samples on steel plates were dried and images were taken.

X-Ray diffractometer (XRD) (Phillips X'Pert Pro) analyses of magnetic particles were studied. Elemental analyses of particles were made by X-ray diffraction. They were irradiated with collimated monochromatic X-rays. The diffraction angle and intensity of diffracted X-rays gave the known several comparable pattern of samples.

Magnetic properties of magnetic particles were examined with the Vibrating Sample Magnetometer (VSM) (LakeShore 7407) at Izmir Institute of Technology .

Atomic Force Microscope (AFM) (Q-Scope 250 Scanning Probe Microscope Ambios. Tech.) analyses of magnetic particles were carried out at the Institute of Solar Energy, Ege University.

Anti-Metadherin (Anti-MTDH) conjugation of D- Penicillamine

Five mg of D-penicillamine was dispersed in 1470 µL of 0.1 M sodium carbonate buffer (pH 9.0). Then, 30 µL of glutaraldehyde was added and mixture was stirred at 4°C for 24 hours. After that, 4 µL of anti-MTDH was added to the mixture, and it allowed to stand overnight at 4 °C.

The solution was centrifugated for five minutes at 10,000 X g by using centrifugal filter units (50,000 NMWL) to remove unbound anti-MTDH .

labeling of anti-MTDH conjugated D- penicillamine

D-PA-anti-MTDH solution: The D-PA-anti-MTDH solution was prepared by dissolving 5 mg of D-penicillamine in freshly prepared 1 mL of 0.1 M sodium carbonate buffer (pH=9.0).

FITC solution: 1 mg FITC was used as labeling agent, and it was dissolved in 1 mL of dimethyl sulfoxide (DMSO).

FITC-D-PA-anti-MTDH: 100 μ L of FITC solution was added into D-PA-anti-MTDH solution at dark condition.

The mixture stayed at 4 $^{\circ}$ C during eight hours in order to label D-PA-anti-MTDH with FITC.

500 μ L of NH_4Cl buffer (50 mM) and FITC-D-PA-anti-MTDH were mixed and stayed at 4 $^{\circ}$ C, during two hours. Then, 100 μ L of glycerine was added. The mixture passed through the column to leave unbound FITC.

Magnetic Nanoparticles Conjugation of FITC Labelled D-Penicillamine-Anti-MTDH

Approximately 1.5 mL of FITC-D-PA-anti-MTDH solution was obtained. 250 μ L of 0.1 M PBS, containing 0.15 M NaCl, 0.005 M EDTA, was added to 1 mL of FITC-D-PA-anti-MTDH solution. After the addition of 2 μ L (150 mg/mL) of magnetic nanoparticles, the mixture was kept at room temperature during 12 hours.

Incorporation Rates of FITC-D-PA-Antibody Conjugated Magnetic Nanoparticles with MCF-7 Cells

MCF-7 breast cancer cell lines were used for this study. The cells were cultured and seeded into the wells of a 24 well culture plate and 9 cm^2 tissue culture petri dishes for fluorescence microscopy, after enough had been produced. In the study of biological activity detection of FITC labelled, magnetic nanoparticles conjugated D-PA-anti-MTDH *in vitro*, exactly 10^5 MCF-7 cells were implanted on petri dishes. Cells were cultured to confluence at 37 $^{\circ}$ C and 5.0% CO_2 D-PA-anti-MTDH-FITC, D-PA-anti-MTDH-magnetic nanoparticles-FITC, control solution and magnetic field applying D-PA-anti-MTDH-magnetic nanoparticles-FITC were used in the study.

Medium over the cells was removed and the cells were washed with PBS for three times. 250 μ L of these samples were put into the wells of a 24 well culture plate and 500 μ L of these samples were put into 9 cm^2 tissue culture petri dishes after washing. NdFeB magnets were placed each well of the plate and magnetic field was applied to each well of these plates, while other well was not under magnetic field. Optimum incubation time was defined for two hours in the study. Culture medium was discarded from the wells at the optimum time (two hours) and washed with PBS for three times. After incubation time, 24 well culture plates were fluorometrically assayed in a multiwell fluorescence plate reader (Thermo, Milford, MA) to determine the %D-PA-anti-MTDH-FITC, D-PA-anti-MTDH-magnetic

nanoparticles-FITC, and magnetic field applying D-PA-anti-MTDH-magnetic nanoparticles-FITC incorporation to cells. 9 cm^2 tissue culture Petri dishes were visualized with 100 X magnification and photographed through epi-fluorescence microscopy (Olympus, Tokyo, Japan). Besides, the magnetic field effect was determined for the several cellular incorporations of the ligands conjugated magnetic particles.

Results and discussion

Structural Properties of Magnetic Particles

SEM Analyses Results

The SEM imaging of magnetic particles is as depicted in Figures 1 to 3. SEM results showed that particles are nearly monodisperse. The average particle size is found to be from 40 to 50 nm. Sizes of the silica coated particles did not change after surface modification with silane.

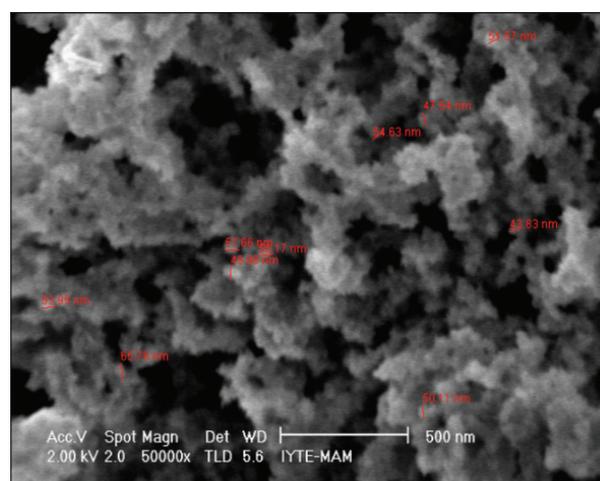


Figure 1. SEM Images of Silica Coated Magnetite.

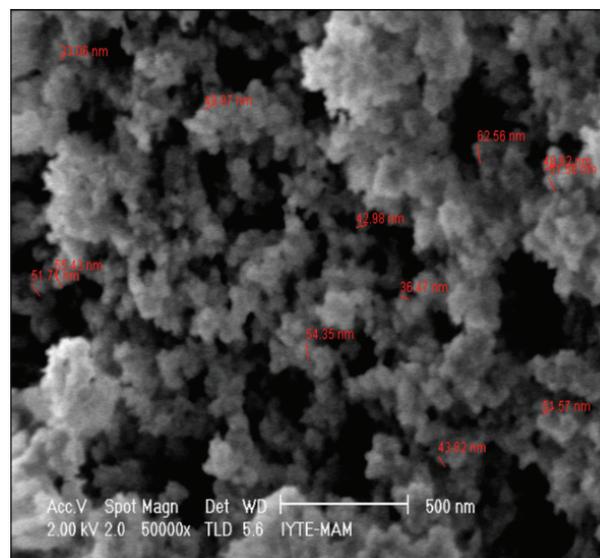


Figure 2. SEM Images of Silanated Magnetite. Nanoparticles with 50000 X magnification.

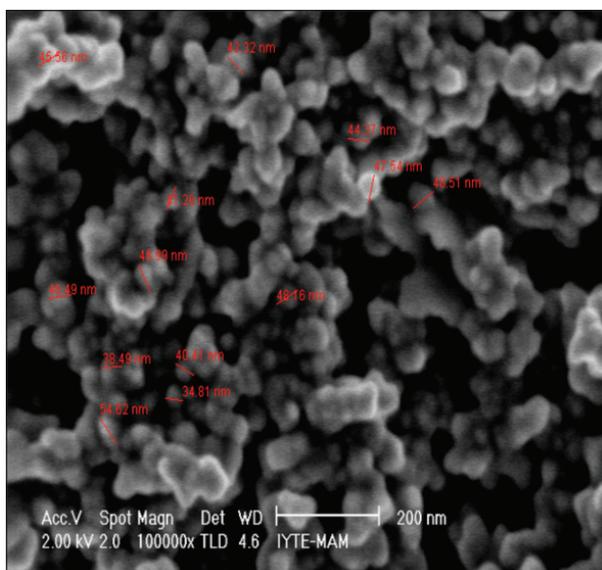


Figure 3. SEM Images of Fe₃O₄ Magnetite. Nanoparticles with 100000 X magnification.

XRD Analyses Results

XRD analyses of magnetic particles, after surface modification, show the X-ray diffraction pattern of the samples paired with Fe₃O₄ diffraction pattern (Figure 4).

VSM Analyses Results

Magnetic properties of magnetic particles were determined with VSM (LakeShore 7407). Magnetization value versus applied magnetic field for Fe₃O₄ magnetic particles was 16.28 emu/g.

AFM Analyses Results

Incorporation Rates of FITC-D-PA-anti-MTDH Conjugated Magnetic Nanoparticles with MCF-7

The obtained D-PA-anti-MTDH-magnetic nanoparticles-FITC complex have been used for *in vitro* imaging of breast cancer cells. The cellular binding efficiency of D-PA-anti-MTDH-FITC and D-PA-anti-MTDH-magnetic nanoparticles-FITC was calculated using the fluorescence signals as a result of the targeted-cell interaction.

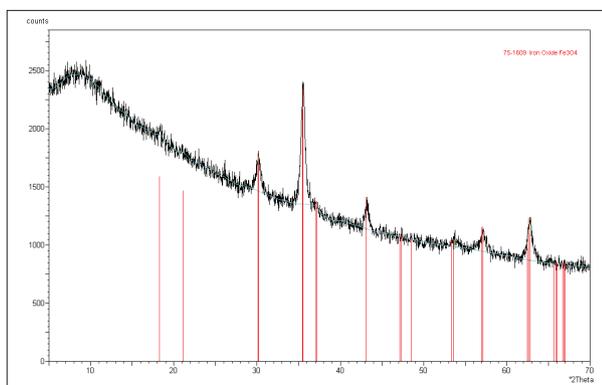


Figure 4. XRD Images of Fe₃O₄ Magnetic Nanoparticles.

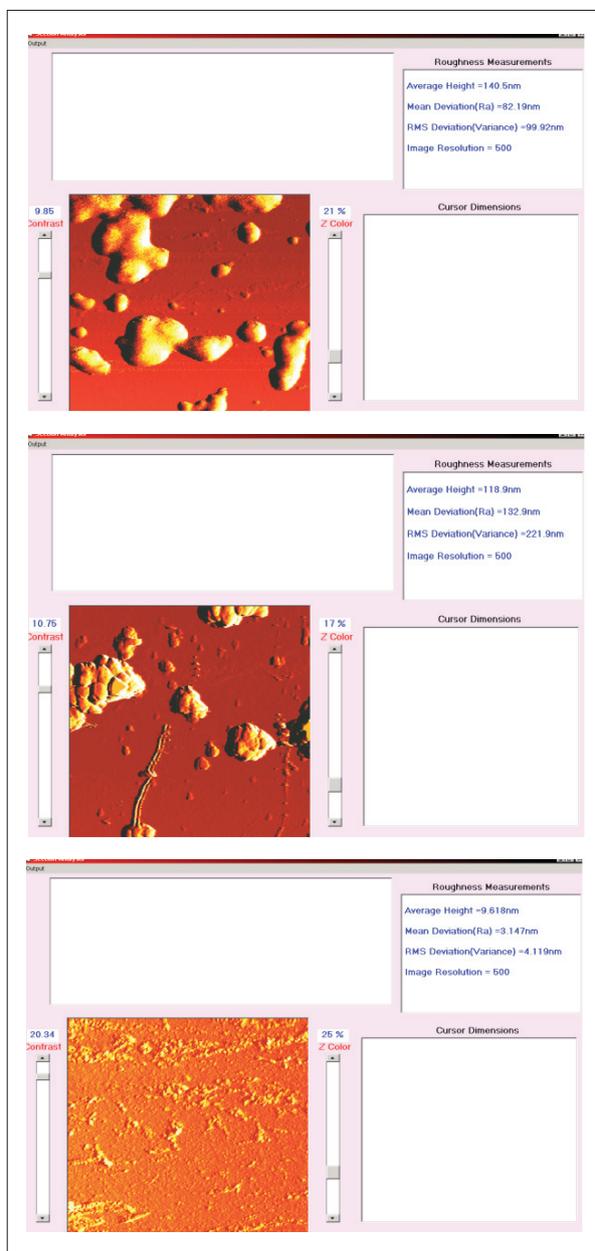


Figure 5. Atomic force microscopy images.

Incorporation on cells of FITC labeled and magnetic nanoparticles conjugated D-PA-anti-MTDH was found higher than FITC labeled D-penicillamine. The results show that magnetic properties and applying magnetic field increased incorporation rates. On the other hand, D-penicillamine throat cancer cells (Detroit) using the same method on cytotoxic effects were shown in another study²³.

D-penicillamine is a good chelating agent for antibody conjugating to nanoparticles. These nanoparticles may be useful as a new class of agents to target antibody or biomolecules for imaging and targeted therapy of cancer. Results of this study are sufficiently encouraging to bring about further evaluation of this and related compounds.

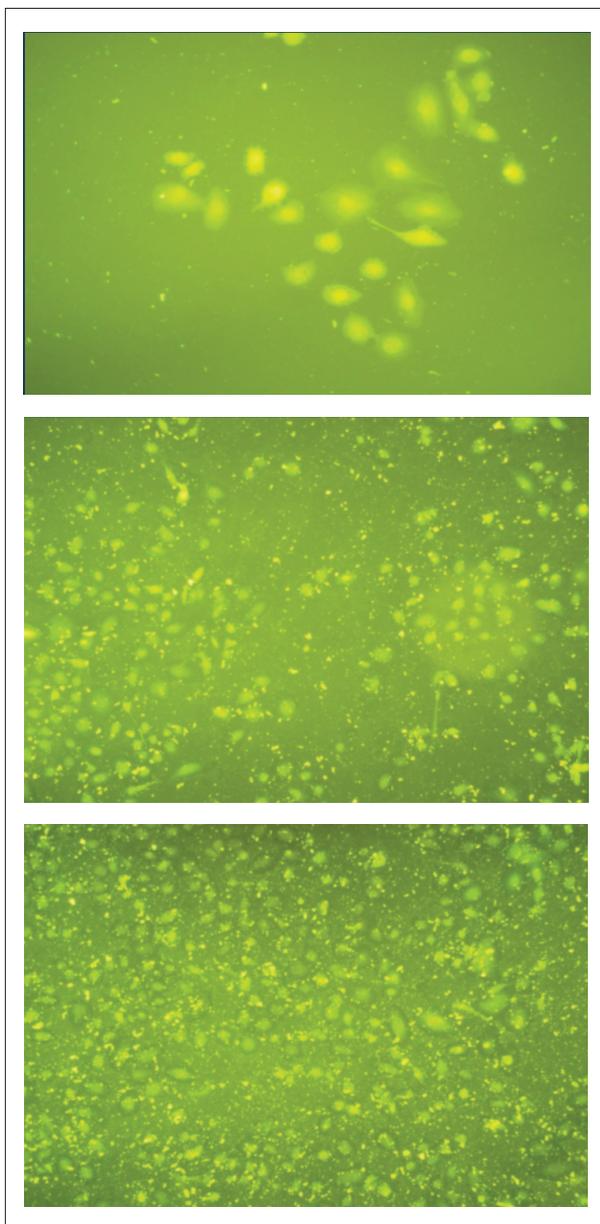


Figure 6. Fluorescence microscopy images of D-penicillamine-(Anti-MTDH)-FITC (A), D-penicillamine-(Anti-MTDH)-FITC-Magnetic nanoparticles (B) and magnetic field applying D-penicillamine-(Anti-MTDH)-FITC-Magnetite nanoparticles (C), after a two-hour incubation with MCF-7 cells with 100 magnifications.

References

- Pope NM, Alsop RC, Chang YA, Smith AK. Evaluation of magnetic alginate beads as a solid support for positive selection of CD34+ cells. *J Biomed Mater Res*. 1994;28:449-57.
- Liu ZL, Ding ZH, Yao KL, Tao J, Du GH, Lu QH, Wang X, Gong FL, Chen X. Preparation and characterization of polymer-coated core-shell structured magnetic microbeads. *J Magn Mater*. 2003;265:98-105.
- Zhou J, Wu W, Caruntu D, Yu MH, Martin A, Chen JF, et al. Synthesis of porous magnetic hollow silica nanospheres for nanomedicine application. *J Phys Chem*. 2007;111:17473-7.
- Pieters BR, Williams RA, Webb C. *Magnetic Carrier Technology*. Oxford, England: Butterworth-Heinemann; 1992.
- Abo M, Chen Z, Lai LJ, Reese T, Bjelke B. Functional recovery after brain lesion - contralateral neuromodulation: an FMRI study. *Neuroreport*. 2001;12:1543-7.
- Edelstein RL, Tamanaha CR, Sheehan PE, Miller MM, Baselt DR, Whitman LJ, et al. The BARC biosensor applied to the detection of biological warfare agents. *Biosensors Bioelect*. 2000;14:805-13.
- Kim CK, Lim SJ. Recent progress in drug delivery systems for anticancer agents. *Pharm Soc Korea*. 2002;25(3):229-39.
- Goodwin S, Peterson C, Hoh C, Bittner C. Targeting and retention of magnetic targeted carriers (MTCs) enhancing intra-arterial chemotherapy. *J Magn Mater*. 1999;194:132-9.
- Liu JW, Zhang Y, Chen D, Yang T, Chen ZP, Pan SY, et al. Facile synthesis of high-magnetization-Fe₂O₃/alginate/silica microspheres for isolation of plasma DNA. *Colloids and Surfaces A. Physicochem Eng Aspects*. 2009;341:33-9.
- Yee Mak S, Hwang Chen D. Binding and sulfonation of poly (acrylic acid) on iron oxide nanoparticles: a Novel, Magnetic, Strong Acid cation Nano-Adsorbent. *Macromol Rapid Commun*. 2005;26:1567-71.
- Lee SY, Harris MT. Surface modification of magnetic nanoparticles capped by oleic acids: characterization and colloidal stability in polar solvents. *J Colloid Interface Sci*. 2006;293:401-8.
- Haddad PS, Martins TM, Souza-Li LD, Li LM, Metzke K, Adam RL, et al. Structural and morphological investigation of magnetic nanoparticles based on iron oxides for biomedical applications. *Mater Sci Eng*. 2008;28:489-94.
- Vande Stat RJ, Muijsers AO, Henrichs AMA, VanderKorst JK. D-Penicillamine: biochemical, metabolic and pharmacological aspects. *Scand J Rheumatol*. 1979;28:13-20.
- Schilsky ML. Wilson disease: genetic basis of copper toxicity and natural history. *Semin Liver Dis*. 1996;16:83-95.
- Acar Ç, Teksöz S, Ünak P, Biber Müftüleri FZ. Investigation Of New Bifunctional Agents: D-Penicillamine. *J Radioanalytical Nuclear Chem*. 2007;273:641-7.
- Horiuchi K, Yokoyama A, Tanaka H, Saji H, Odori T, Morita R, et al. Technetium coordination state as a factor of stability in ^{99m}Tc-complexes used in hepatobiliary system: comparative studies on ^{99m}Tc-complexes of prididoxal with glutamate (Tc-PG) and isoleucine (Tc-PI). *Eur J Nucl Med*. 1981;6:573-9.
- Unak P, Tunç M, Duman Y. Labeling of Penicillamine di sulfide with technetium-99m. *Appl Rad Isot*. 1998;49(7):805-9.
- Tröger W, The Isolde Collaboration. Hg(II) Coordination Studies in Penicillamine Enantiomers by ^{199m}Hg-TDPAC. *Hyperf Int*. 2001;136/137:673-80.
- Brown DM, Ruoslahti E. Metadherin, a cell surface protein in breast tumors that mediates lung metastasis. *Cancer Cell*. 2004;5(4):365-74.
- Sutherland HG, Lam YW, Briers S, Lamond AI, Bickmore WA. 3D3/lyric: a novel transmembrane protein of the endoplasmic reticulum and nuclear envelope, which is also present in the nucleolus. *Exp Cell Res*. 2004;294(1):94-105.
- Hafeli UO, Sweeney SM, Beresford BA, Humm JL, Macklis RM. Effective Targeting of Magnetic Radioactive ⁹⁰-Y-Microspheres to Tumor Cells by an Externally Applied Magnetic Field. Preliminary In Vitro and In Vivo Results. *Nucl Med Biol*. 1995;22:147-55.
- Mohapatra S, Mallick SK, Skgghosh T, Pramanik P. Synthesis Of Highly Stable Folic Acid Conjugated Magnetite Nanoparticles For Targeting Cancer Cells. *Nanotechnology*. 2007;18(38):385102.
- Gülçüler G, Yıldır A, Hepsöğütü B. İNEPO 17. Uluslararası Çevre Proje Olimpiyatı; 2009.

